Brief Report

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Development of a silkworm infection model for evaluating the virulence of *Mycobacterium intracellulare* subspecies estimated using phylogenetic tree analysis based on core gene data

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SUMMARY Non-tuberculous mycobacteria (NTM) cause skin infections, respiratory diseases, and disseminated infections. *Mycobacterium avium* and *Mycobacterium intracellulare*, which are slow grown *Mycobacterium*, are main agents of those NTM diseases. A silkworm infection model with *Mycobacterium abscessus*, a rapidly growing *Mycobacterium* species, was established to quantitatively evaluate its virulence within a short period. However, a silkworm infection model to quantitatively evaluate the virulence of *M. intracellulare* has not yet been developed. In this study, we determined the virulence of *M. intracellulare* subspecies within 4 days using a silkworm infection model. The subspecies of *M. intracellulare* strains used in this study were estimated by phylogenetic tree analysis using core gene data. The median lethal dose (LD_{50}) values, which are the dose of a pathogen required to kill half of the silkworms in a group, were determined 4 days after infection. The LD_{50} value of *M. intracellulare* subsp. *chimaera* DSM44623 was higher than that of *M. intracellulare* subsp. *intracellulare* ATCC13950. These results suggest that the virulence of *M. intracellulare* subspecies can be compared using a silkworm model within 4 days.

Keywords *Mycobacterium intracellulare*, subspecies, silkworm, infection, virulence

1. Introduction

Non-tuberculous mycobacteria (NTM) are classified as mycobacteria, excluding *Mycobacterium tuberculosis* and *Mycobacterium leprae* (*1,2*). NTM causes severe chronic diseases such as respiratory infections in immunocompromised humans (*1–4*). *Mycobacterium avium* and *Mycobacterium intracellulare* are mainly isolated from the patients with NTM diseases (*5*). Therefore, *M. avium* and *M. intracellulare* may be highly virulent species in the NTM. Infection experiments using mammals such as mice are useful for evaluating the virulence of these bacteria (*6,7*). However, several weeks are needed for mice to die from infection, and it is difficult to conduct infection experiments using a large number of mice from the viewpoint of animal welfare.

Silkworm, an invertebrate, is useful as an alternative animal for evaluating the virulence of pathogens (*8*). Because silkworms have fewer ethical issues than mammals, a large number of silkworms can be used for infection experiments (*9*). Therefore, silkworm infection models are used to identify virulence-related genes by isolating avirulent mutants from gene-disrupted libraries (*10–13*). Using silkworm infection models, the virulence of pathogens was determined by calculating the median lethal dose (LD_{50}), which is the dose of a pathogen required to kill half of the animals in a group (*14–17*). Moreover, the LD_{50} values of *Mycobacterium abscessus* clinical strains were determined, and the virulence of the *M. abscessus* clinical strains was quantitatively compared (*14*). Furthermore, the silkworm models with mycobacteria such as *M. abscessus*, *M. avium*, or *M. intracellulare*, were developed for evaluating the efficacy of anti-mycobacterial compounds (*14,18,19*). However, quantitative evaluation of the virulence of *M. intracellulare* strains using silkworms has not been performed.

The classification of *Mycobacterium intracellulare* has changed significantly over the past decade. Based on genome information and Average Nucleotide Identity (ANI) analysis, *M. intracellulare* subsp. *yongonense* was integrated into *M. intracellulare* subsp. *chimaera*, and *M. paraintracellulare* was integrated into *M. intracellulare* subsp. *intracellulare*. Currently,

the subspecies of *Mycobacterium intracellulare* are identified as *M. intracellulare* subsp. *intracellulare* and *M. intracellulare* subsp. *chimaera*. Sequencing of the 16S rRNA gene is useful for identifying bacterial species (*20,21*). Because the 16S rRNA gene is highly similar among *Mycobacterium* species, sequencing analysis is insufficient to distinguish closely related subspecies (*22*). *M. avium* subspecies such as *avium*, *hominissuis*, *paratuberculosis*, and *silvaticum* are determined by sequencing the insertion sequences and the internal transcribed spacer 1 region of rRNA genes (*23*). On the other hand, sequencing analysis of the insertion sequences and the internal transcribed spacer 1 region of rRNA genes is not enough to distinguish species/subspecies closely related to *M. intracellulare* (*23*). Even with MALDI TOF-MS, which has been the mainstream method for identification in recent years, it is not possible to distinguish between these two subspecies. Therefore, the development of a novel method for estimating *M. intracellulare* subspecies is desired.

In the present study, we estimated the subspecies of *M. intracellulare* strains by phylogenetic tree analysis based on the core gene data of *M. intracellulare*. The LD_{50} values of the *M. intracellulare* strains were determined using silkworms. These findings suggest that the silkworm infection model is useful for quantitatively calculating the virulence of *M. intracellulare* strains.

2. Materials and Methods

2.1. Phylogenetic tree analysis

Core gene-based phylogeny in *M. intracellulare* strains from the National Center for Biotechnology Information (NCBI) database was generated following the pipeline described by Atxaerandio-Landa *et al*. (*24*). Specifically, 113 assemblies (deposited as *M. intracellulare*, *M. intracellulare* subsp. *chimaera*, *M. intracellulare* subsp. *yongoense*, or *M. paraintracellulare*) were downloaded from the NCBI database with ncbi-genome-download v0.3.1 (accessed on 22 April 2024) and assessed using CheckM2 v1.0.1 (*25*). The 112 *M. intracellulare* sequences were assessed as exhibiting > 99% completeness and < 2% contamination and were annotated using Prokka v1.14.6 (*26*), and general feature format (gff) files were produced. The gff files were analyzed for core genes using Roary v3.13.0 (*27*). The core alignment was trimmed using trimAl v1.4. rev15 with the option '-automated1' (*28*). A maximum likelihood tree was constructed from the alignment composed of 3182 core genes (3,137,717 bp) using the best-fitted nucleotide substitution model (GTR+ F + I + R4) in IQ-TREE v2.2.2.7 (*29*) and visualized using the Interactive Tree of Life (iTOL) (*https://itol.embl.de/*). The core genes were selected based on the criteria that the BLASTp cut-off value was set at 95% according to a previous report (*30*).

2.2. Reagents

Middlebrook 7H9 broth, Middlebrook 7H10 agar, and Middlebrook OADC enrichment were purchased from Becton, Dickinson, and Company (Sparks, MD, USA). Middlebrook 7H9 broth and Middlebrook 7H10 agar were supplemented with 10% Middlebrook OADC Enrichment.

2.3. Bacterial strain and culture condition

M. intracellulare strains were used in this study (Table 1). The *M. intracellulare* strains were grown on Middlebrook 7H10 agar plates at 37°C. A single colony was then inoculated into 5 ml of Middlebrook 7H9 broth and incubated at 37°C for 5 days.

2.4. LD_{50} determination using a silkworm infection model

Silkworm infection experiments with *M. intracellulare* were performed according to a previous study with slight modifications (*14*). Fifth-instar larvae were reared on an artificial diet (Silkmate 2S; Ehime-Sanshu Co., Ltd., Ehime, Japan) for 24 h. *M. intracellulare* cells grown in Middlebrook 7H9 broth were collected by centrifugation and resuspended in sterile saline. A 50-µL sample solution was administered to the silkworm hemolymph by injecting the silkworm dorsally using a 1-ml tuberculin syringe (Terumo Medical Corporation, Tokyo, Japan). The LD_{50} values were determined according to a previous study with slight modifications (*14*). *M. intracellulare* cells grown in Middlebrook 7H9 broth were resuspended in saline. A 2- or 4-fold dilution series of bacterial suspensions was prepared. The bacterial suspension ($2.2 \times 10^5 – 9.6 \times 10^7$ cells/50 µL) was injected into the silkworm hemolymph, and the silkworms were incubated at 37˚C with an artificial diet, Silkmate 2S. The number of surviving silkworms was counted at 4 days after infection. LD_{50} values were determined from the data of three or four experiments using a simple logistic regression model in Prism 9 (GraphPad Software, LLC, San Diego, CA, USA, *https://www.graph pad.com/scientific-software/prism/*).

Table 1. LD_{50} values of *M. intracellulare* strains in a silkworm infection model

Strains	LD_{50} (x 10^7 cells/larva)
M. intracellulare subsp. *intracellulare* ATCC13950	2.1
M. intracellulare subsp. *intracellulare* MOTT64	5.6
M. intracellulare subsp. *chimaera* DSM44623	> 9.6
M. intracellulare subsp. *chimaera* Asan36527	2.1

3. Results and Discussion

We estimated the subspecies of *Mycobacterium* strains (ATCC13950, MOTT64, DSM44623, and Asan36527) used in this study based on phylogenetic tree analysis of core gene sequences. The *M. intracellulare* subsp. *intracellulare* cluster including ATCC13950 and the *M. intracellulare* subsp. *chimaera* cluster including DSM44623 were distinctly separated (Figure 1). Therefore, phylogenetic tree analysis based on core gene

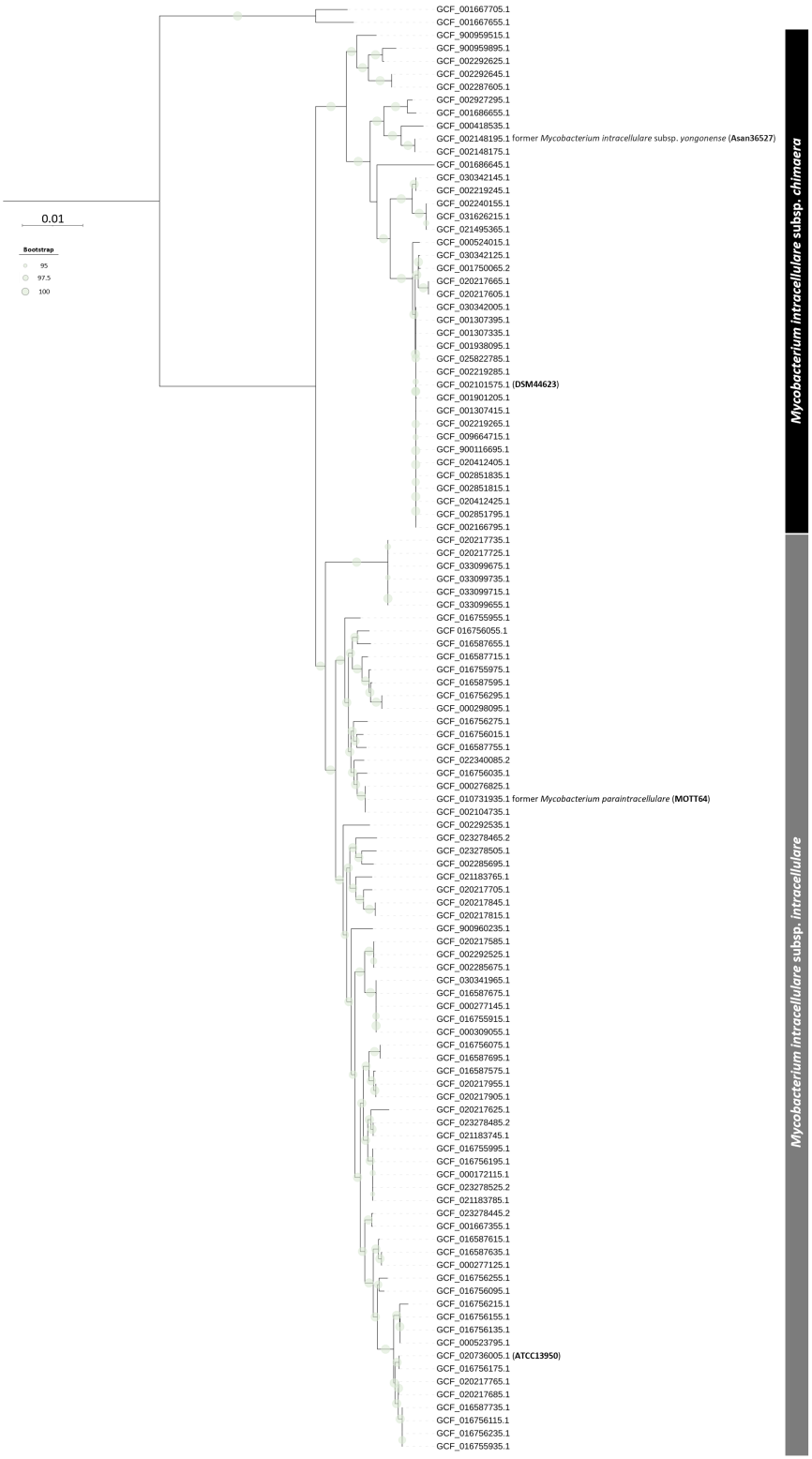

Figure 1. Phylogenetic tree of *M. intracellulare* subspecies strains. Maximum likelihood tree based on 3,182 core genes from 112 strains of *Mycobacterium intracellulare* identified with Roary v3.13.0. The tree was generated using the best-fitted nucleotide substitution model (GTR+ F + I + R4) in IQ-TREE v2.2.2.7, with a 1,000-replicate, ultra-fast bootstrap approximation. Scale bar represents number of nucleotide substitutions per site. The tree is midpoint rooted.

sequences revealed two subspecies; *M. intracellulare* subsp. *intracellulare* and *M. intracellulare* subsp. *chimaera*. MOTT64 is included in the *M. intracellulare* subsp. *intracellulare* cluster (Figure 1). On the other hand, Asan36527 was included in the *M. intracellulare* subsp. *chimaera* cluster (Figure 1). These results suggest that MOTT64 and Asan36527 strains belong to *M. intracellulare* subsp. *intracellulare* and *M. intracellulare* subsp. *chimaera*, respectively.

Next, we established a silkworm infection model to determine the LD_{50} of *M. intracellulare*. ATCC13950 is a type strain of typical *M. intracellulare* subsp. *intracellulare* (*6*). A sample solution of serially diluted ATCC13950 cells ($5.6 \times 10^5 – 3.3 \times 10^7$ cells per larva) was injected into silkworms, and the injection of a large number of bacterial cells led to silkworm death (Figure 2A). The LD_{50} value of ATCC13950 in the silkworm infection model was 2.1×10^7 cells/silkworms (Table 1). These results suggest that a silkworm infection model was established to determine the LD_{50} value of the *M. intracellulare* type strain. We next compared their virulence by determining the LD_{50} values of *M. intracellulare* strains using the silkworm infection model. The LD_{50} values of MOTT64, DSM44623, and Asan36527 were 5.6, > 9.6, and 2.1×10^7 cells/silkworms, respectively (Figures 2B-2D) (Table 1). The LD_{50} values of DSM44623 were higher than those of other strains (Table 1). These results suggest that the virulence of *M. intracellulare* strains was quantitatively calculated based on their LD_{50} values. The silkworm infection model can determine the virulence of the *M. intracellulare* subspecies. In a previously reported silkworm infection model, silkworms were not fed after injection of the sample solution, and saline-injected silkworms died within 4 days (*19*). In this study, silkworms were fed after injection, and the saline-injected silkworms survived for more than 4 days. Therefore, we used the conditions under which the LD_{50} value of the type strain of *M. intracellulare* was calculated after four days of infection. Using these experimental conditions, the LD_{50} values of *M. intracellulare* subspecies were determined. *M. intracellulare* subsp. *chimaera* DSM44623 were lower silkworm killing ability than other strains used in this study. On the other hand, the LD_{50} values for *M. intracellulare* subsp. *intracellulare* ATCC13950 and *M. intracellulare* subsp. *chimaera* Asan36527 were the same. Therefore, the virulence of *M. intracellulare* subsp. *intracellulare* ATCC13950 and *M. intracellulare* subsp. *chimaera* Asan36527 is similar. We assumed that the virulence of each *M. intracellulare* strain was different rather than clear differences in virulence between subspecies of *M. intracellulare*. Revealing the genome structure differences between highly virulent and avirulent strains will be important.

In conclusion, we established a silkworm infection model to compare the virulence of *M. intracellulare* subspecies estimated by phylogenetic tree analysis using core gene data. Using the silkworm infection model, the virulence of *M. intracellulare* strains can be determined within 4 days. We assumed that these experimental methods might contribute to the comparison of the virulence of *M. intracellulare* strains.

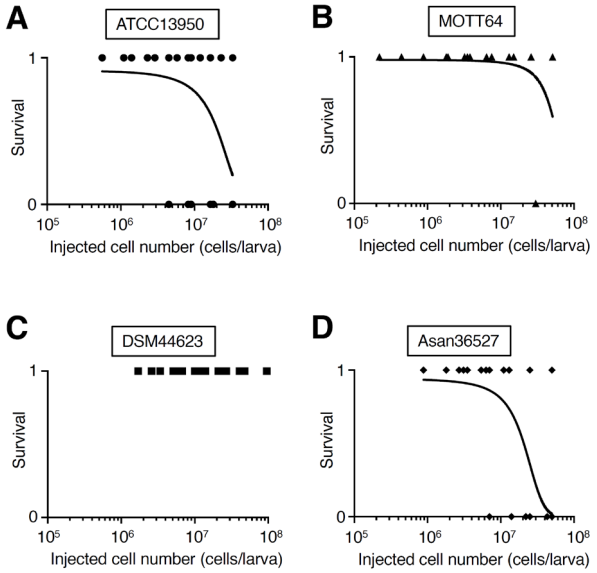

Figure 2. Infection experiments of *M. intracellulare* strains against silkworms. Silkworms were injected with saline or *M. intracellulare* cells ($2.2 \times 10^5 – 9.6 \times 10^7$ cells/silkworm). ATCC13950 strain: $5.6 \times 10^5 – 3.3 \times 10^7$ cells/silkworm. MOTT64 strain: $2.2 \times 10^5 – 5.1 \times 10^7$ cells per silkworm. DSM44623 strain: $1.7 \times 10^6 – 9.6 \times 10^7$ cells/silkworm. Asan36527 strain: $8.8 \times 10^5 – 5.0 \times 10^7$ cells/silkworm. The silkworms were incubated at 37°C for 4 days with an artificial diet. The number of surviving silkworms was counted at 4 days after infection.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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